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Anti-cancer Properties of Protein Hydrolysate from the Posterior Salivary Gland of *Amphioctopus membranaceus* (Quoy & Gaimard, 1832)

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Abstract
The present study was carried out to evaluate the anti-cancer properties of protein hydrolysate from posterior salivary gland (PSG) of *Amphioctopus membranaceus.* The sample (octopus) was initially screened for species identification through CO-I gene. The PSG of the animal was homogenized and centrifuged at 6000 rpm for 10 min. Further, enzymatic hydrolysis was performed using papain enzyme at a dose of 2.5% w/w. Later the hydrolysate (supernatant) obtained was subjected to fractionation using gel permeation chromatography. 20 fractions were collected and the fraction 4 (OM-H) was found to have the maximum amount of protein content of 3.2 mg/mL. SDS-PAGE depicted prominent bands ranging from 14 to 25 kDa. The IC_{50} evaluation was studied using Vero cell lines that showed the cytotoxic effect of OM-H at 405 µg/mL and in HeLa cell lines 250 µg/mL showed the highest inhibition rate of 45.01%. Thus, the study showed that partially purified PSG hydrolysate was found to display good anti-cancer activity against human cancer cell lines.

Keywords Cephalopods · Cytotoxicity · Enzymatic hydrolysis · Posterior salivary gland (PSG) · SDS-PAGE

Introduction

The marine environment with its diversification is rich in flora and fauna including all marine organisms in the earth's ecosystem. Marine organisms are used completely or partly to synthesize products and to enhance the lifestyle of plants, animals and microorganisms. The marine environment may contain over 80% of the world's plant and animal species (McCarthy and Pomponi 2004). Therefore, plenty of marine flora and fauna species are yet to be screened for the discovery of useful targeted antibiotics. Majority of the anticancer agents derived from the marine molluscs have shown good potential against tumor activity. The search for new products from the marine organisms has been consistent to this day of research in the marine ecosystem. Therefore, bioactive compounds from the marine habitat have been represented as the under-exploited source for potentially active pharmaceutical agents. Hence, marine produce a variety of metabolites, some of which can be used for drug development (Chellaram et al. 2010). Cancer, in general, is the collection of various diseases due to abnormal cell growth.

Normally, cancer cell starts to replicate and spread to surrounding areas due to their malignancy forming malignant tumours, whereas benign remains confined to a particular area. Cancer is regarded as the largest cause of death in the whole world. Chemotherapy is the most performed treatment in recent times, which uses chemotherapeutic agents as a standard to block the development of cancer in humans. In 2007, trabectedin (Yondelis; PharmaMar) became the first marine anticancer drug to be approved in the European Union (Molinski et al. 2009). Venoms used as antitumor agents serve as a major contributor in cancer therapy with the use of protein, peptides and enzymes originating from animals of different species. Some of the snake venom proteins or peptides and enzymes when purified and evaluated may bind exclusively to cancer cell membranes, affecting the migration and proliferation of these cells (Vyas et al. 2013). Since the snake venom shows the highest cytotoxic potential, the ophidians accidentally cause a large amount of

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tissue damage, signifying a gifted utilization of these venoms or their components as anti-tumor agents (Calderon et al. 2014).

Similarly, in snake venoms, phospholipase A2 that catalyzes the hydrolysis of Sn-2 position of phospholipids releasing fatty acids and lysophospholipids are connected to a broad spectrum of biotechnological activities (Juliana et al. 2016). In addition to this, bee venom has also been proven to have anti-proliferative effects in vitro and reducing tumor growth in vivo (Orsolic et al. 2003). However, the snake venom is considered desirable due to its high cytotoxicity property. Majorly, venoms showing cytotoxicity to malignant cells are cytotoxic to normal cells as well (Abu-Sinna et al. 2003). Therefore, under this class of marine fauna Octopus has been explored for potential bioactive compounds. Except for the small molecular drug, there are various anti-cancer drug modalities, including protein drugs such as antibody, peptide, nano-medicine and gene medicine (Zsi Zhang et al. 2017) studied so far. In addition, cephalopods ink was used in the development of drugs and biomedical application (Naraoka et al. 2000).To introduce and discover targeted antibiotics against cancer agents; a new approach was adapted here, where the anticancer activity has been studied against human cancer cell lines.

Thus, for our study, we considered *A. membranaceus,* a mild toxic venomous cephalopod *A. membranaceus* is benthic, which resides in ranges about 60 m in-depth, in tropical and subtropical waters. The maximum mantle length of the species is about 8 cm and total about 30 cm. Also, these members from *Amphioctopus* are gonochoric, resides in tropical and subtropical waters, found in Pacific and Indian oceans but rarely found in Atlantic. The venom gland of *A. membranaceus* consists of both anterior and posterior salivary gland associated with buccal mass and mandible. The venom from these glands consists of a potent neurotoxin called 'Maculotoxin' later named as 'Tetrodotoxin' which was first identified in *H. maculosa* (Sheumack et al. 1978). Of these two salivary glands, the anterior glands which are generally associated with mucous production, and the posterior glands are considered venomous due to the presence of numerous bioactive substances such as tyramine (Henze 1913), histamine (Botazzi and Valentini 1992), octopamine and enteramine (Erspamer and Asero 1953). Generally, the genes for toxin production are common to all octopuses and probably all the members of the group are considered venomous to some extent (Fry et al. 2009). However, the toxicity level may vary accordingly in different species. With respect to the current study, cephalopod such as *Octopus dofleini* has been known to produce active protein that is toxic against crustaceans (Songdahl and Shapiro 1974). In the present investigation, an attempt was made to hydrolyze the posterior salivary gland using an enzyme called papain (cysteine protease). Simultaneously, anticancer properties of the fractionated posterior salivary gland (PSG) hydrolysate of *A. membranaceus* against the HeLa cell line (cervical cancer cell line) was tested along with their protein profiles.

Methodology

Sample Collection

Octopus (*A. membranaceus)* samples used in this study were collected from Pazhayar landing center (Lat 11 21′ 32″ N; Long 79 49′ 25″ E) situated on the southeast coast of India. The collected samples were brought to the laboratory and were preserved in the refrigerator for further use. Total of 56 species were dissected and the Posterior Salivary Glands (PSG) was removed aseptically. The individual PSG weighed 0.86 g. Further, the sample/s was/were subjected to species identification and enzymatic hydrolysis.

Extraction, Amplification and Sequencing of Target Octopus Species

The total genomic DNA of the sample was extracted using Phenol Chloroform method standardized by CAGL. Quality of the genomic DNA was assessed using 0.7% agarose gel along with 1kb DNA ladder as size standard and the quantity of the genomic DNA was assessed in bio photometer (Eppendorf). Amplification of CO-I gene (709bp) was carried out using Universal forward (GGTCAACAAATCATAAAGATATTGG) and reverse (TAAACTTCAGGGTGACCAAAAAATCA) primers. PCR generated amplicons were confirmed by running the samples on 2% agarose gel along with 100bp DNA ladder. PCR products were subjected for purification using Gene JET PCR purification kit (Thermo Scientific, EU-Lithuania) to remove the primer-dimer and other carryover contaminations. The quality of the purified PCR product was assessed using 2% agarose gel and was found to be good for sequencing. Amplified PCR product was purified and prepared for Cycle sequencing using the Big Dye® Terminator 3.1 sequence kit (Applied Biosystems, Foster City, California, USA). After cycle sequencing, the product was purified using Ethanol-EDTA purification protocol to remove the un-incorporated dNTP's, ddNTP's and primer-dimer. The purified cycle sequencing products were dissolved in 12 µL Hi-Di formamide and the sample was subjected for denaturation at 95 °C for 5 min. Denatured products were subjected for sequencing in forward and reverse directions using Genetic Analyzer 3500 (Life Technologies Corporation, Applied Biosystems®, California 94404, USA) as per manufacture's instruction. Sequences were aligned and edited using Mega software version 6 (Tamura et al. 2013) to confirm the species.

Enzymatic Hydrolysis

Frozen *A. membranaceus* posterior gland was thawed, minced to homogenate was centrifuged at 6000 rpm for 10 min at 4 °C. The supernatant obtained was mixed with sodium phosphate buffer (pH 6) with a liquid-to-solvent ratio of 1:1 (v/v) for 24 h with continuous stirring along with papain (HiMedia, purified from papaya latex) at pH 6.0, 37 °C with a total enzyme dose of 2.5% considering the substrate. Enzymatic hydrolysis was arrested by heating for 10 min in boiling water and the hydrolysate was centrifuged at 10,000 rpm for 15 min (Fan et al. 2012) with slight modifications. The supernatant was collected and lyophilized for fractionation through Gel permeation chromatography.

Fractionation of Hydrolysate by Gel Permeation Chromatography

Hydrolyzed sample 300 mg/3mL was injected onto a manually prepared Sephadex-G25 (Sigma) column (1.6 × 15 cm) that was pre-equilibrated with deionized water and stepwise eluted with 60 mL of the same. The bound proteins were eluted at a flow rate of 1 mL/min and the absorbance was measured at 280nm to determine the elution profile. 3 mL of each fraction was collected, lyophilized and later its protein content was measured.

Protein Estimation for OM-H

Total protein content of the lyophilized fraction was determined by the modified method of Lowry et al. (1951) Different dilutions of bovine serum albumin (BSA, HiMedia) were prepared by mixing stock BSA solution (1 mg/mL) and water in a test tube. The BSA range was 0.2 to 1.0 mg/mL. Then, 0.1 mL of aliquots of standard protein solution and samples were transferred to different test tubes, followed by the addition of 0.5 mL of Lowry's reagent (Reagent A, manually prepared for laboratory use) and vortexed. The solution in each tube was then added 4.0 mL of Folin–Ciocalteau (Reagent B, Qualigens) and incubated in dark conditions at room temperature for 15 min. The absorbance was measured at 660 nm (Molecular Devices, SpectraMax) and the absorbance against the standard concentration of BSA was plotted to obtain a standard calibration curve, and the protein contents of the samples were determined based on the standard curve plotted above.

Anti-cancer Activity for OM-H Using MTT Assay

The HeLa cell line (Cervical Cancer Cell line) was plated using 96 well plates at a concentration of 1×10^5 cells/well in DMEM media in 1X Antibiotic Solution and 10% fetal bovine serum (Himedia, India) and kept in a CO_2 incubator at 37 °C with 5% CO_2. The cells were washed with 200 µL of 1X PBS, and then the cells were treated with various test concentrations of the compound in serum-free media and incubated at 24 h. The medium was aspirated from cells at the end of the treatment period. 0.5 mg/mL MTT prepared using 1X PBS was added to the cells and incubated at 37 °C for 4 h in a CO_2 incubator. After the incubation period, the medium containing MTT was discarded from the cells and washed using 200 µL of PBS. The crystals formed were dissolved in 100 µL of DMSO and thoroughly mixed. The development of colour intensity was evaluated at 570 nm (Mosmann 1983) and percentage inhibition was calculated.

Results

Species Identification Using Cytochrome Oxidase-1 Sequencing

Quality of the genomic DNA was assessed using 0.7% agarose gel along with 1kb DNA ladder as size standard shown in Fig. 1. PCR-generated amplicons (709 bp) were confirmed by running the samples on 2% agarose gel along with a 100 bp DNA ladder. Amplified PCR product was purified sequenced and aligned using Mega software version 6 and the species confirmed as *Amphioctopus membraneous* and accession number was given KF413892.1. The FASTA sequence obtained was constructed as a phylogenetic tree in terms of species and distance variation provided below (after alignment and editing) of Mitochondrial COI gene showing in Fig. 2a, b.

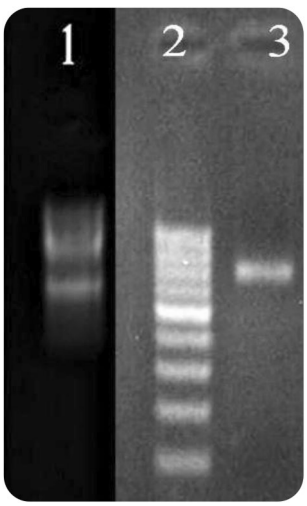

Fig. 1 Lane 1: isolated octopus DNA; Lane 2: 100bp DNA marker, Lane 3: COI gene of Octopus sample

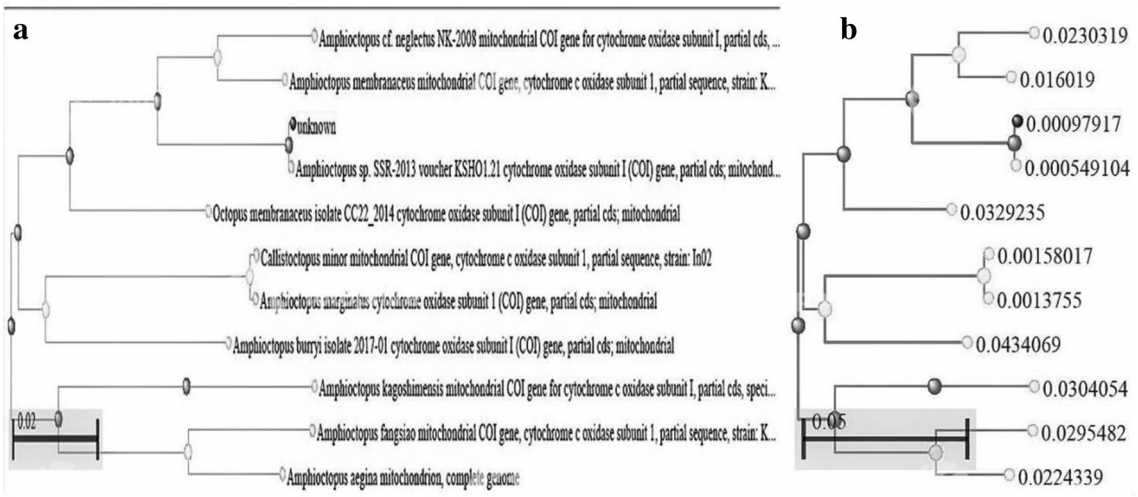

Fig. 2 **a**, **b** Neighbor-joining tree based on CO-I sequences showing the relationship between *A. membraneous* (709 nucleotides) and 10 species

Enzymatic Hydrolysis and Fractionation by Gel Permeation Chromatography (GPC)

After the enzymatic hydrolysis, a total 3 mL hydrolyzed sample was taken and it was then subjected for fractionation in gel permeation chromatography (column fractionation), where 20 fractions (F) of 3 mL volume each were collected and was observed at 280 nm. In this, as a result, two peaks were seen as F4 and F8 fraction, where F4 signifies the highest peak labelled as OM-H shown in Fig. 3 and its total protein content was estimated. Then the molecular weight of the same fraction was determined by SDS-PAGE.

Protein Estimation for OM-H

After repeated fractionation through GPC, fraction 4 (OM-H) was found to have a high protein content as it showed higher absorbance (0.964 O.D) at 280 nm. Further, fractionation was collected and lyophilized. The protein concentration was estimated to be 3.2 mg/mL.

Molecular Weight Determination of OM-H

The electrophoretic profile was obtained by staining OM-H using coomassie brilliant blue dye as shown in Fig. 4. The molecular weight of the crude and fractionated hydrolysate from *A. Membraneous* was estimated by 12% gel. The crude hydrolysate showed bands near 20, 25 and 43 kDa whereas OM-H showed bands near 20 and 25 kDa when compared using standard protein molecular weight markers ranging from 29 to 205 kDa respectively.

Anti-cancer Activity of OM-H

The anticancer activity which was studied in HeLa cell lines (cervical cancer cell lines) showed the prominent result as when the sample (OM-H) was introduced in the Vero cell lines, it showed cytotoxic effect with an IC_{50}value of 405 µg/mL, which indicated that the Vero cells are able to show cell 50% viability up to 405 µg range, which further suggested that the sample can be tested for activity up to this range in HeLa cell lines. When studied in HeLa cell lines

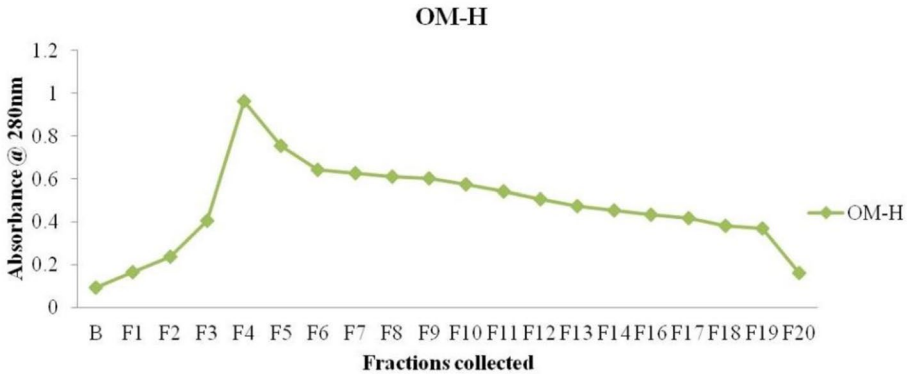

Fig. 3 Graph showing different absorbance range of purified fractions from blank to F20 of the hydrolyzed sample of *A. membranaceus*

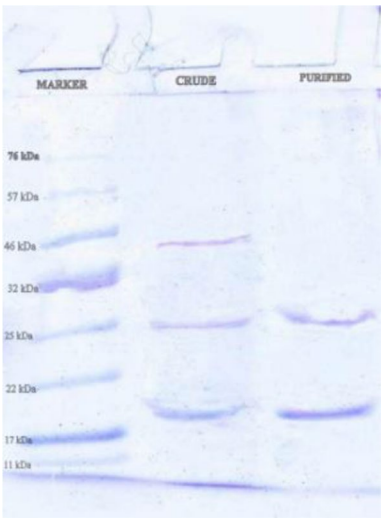

Fig. 4 Shows the electrophoresis profile of OM-H through SDS-PAGE

using different concentrations i.e., control, 150 µg/mL, 200 µg/mL and 250 µg/mL, variations were observed due to an increase in cytotoxicity to HeLa cells. The data depicted as, control 99.85%, followed by 16.03% and 32.08% of cell death at sample concentrations of 150 µg/mL and 200 µg/mL respectively. The highest concentration was observed at concentration of 250 µg/mL showing 45.01% of cytotoxicity as shown in Fig. 5a–c.

Discussion

Anticancer protein or peptides have characteristics of multi-function, high sensitivity, stability and so on. Many peptides and proteins from marine molluscs possess anti-cancer activities. As earlier, Dolastatin 10, isolated from sea hare *D. auricualaria* has entered into clinical trails. Dolastatin 10, is a pentapeptide with four of the residues being structurally matchless and considered to be the most potent anti-proliferative agent against murine PS leukemia cells from marine origin (Pettit et al. 1987). Xiang Xio et al. reported anticancer glycopeptides from *Merettix meretrix* and its inhibitory rate affecting the KB cell line was 69% at 200 µg/mL. In the current study, the protein hydrolysate of *A. membraneous* was obtained by enzymatic hydrolysis catalysed by enzyme papain at specific conditions (pH 6 and 37 °C) and the reaction was stopped after 24 h of incubation. Whereas, in another study same conditions as above used for papain, where the incubation time for that particular study was for 3 h (Fan et al. 2012). Likewise, enzymatic hydrolysis was performed using the three proteases protamex, alcalase, and flavourzyme by the pH-stat method (24 h, pH 8, and 50 °C). Three fractions were generated namely insoluble sludge, a

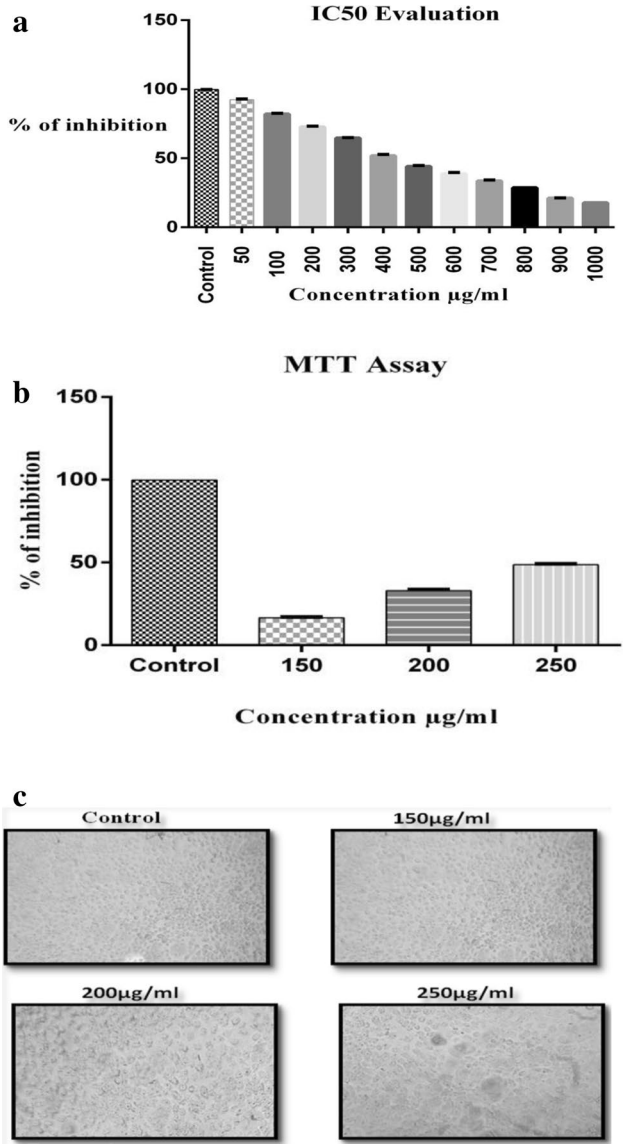

Fig. 5 **a** Cytotoxic IC_{50} value determination of OM-H on Vero cell line. Values were presented as mean ± SEM, statistical significance was performed by one-way ANOVA followed by Tukey's multiple comparison tests. **b** Anticancer activity of OM-H on HeLa cell line. Values were presented as mean ± SEM, statistical significance was performed by one-way ANOVA followed by Tukey's multiple comparison tests. **c** Showing the Anti-cancer activity of OM-H on HeLa cancer cell line

soluble aqueous phase, and an oily phase. For each fraction, lipids, phospholipids and proteins were quantified (Emna et al. 2009).

In our current study, only the protein content was quantified after fractionation since the target molecule was only protein. Ding et al. (2011) hydrolyzed the sepia ink with trypsin to obtain peptides; hydrolysates were isolated by ultrafiltration and purified using G-25 gel filtration. The purity of *sepia* ink oligopeptides was evaluated by HPLC

and its peptide sequence was detected. Umayaparvathi et al. (2014) fractionated hydrolysate on Sephadex G-25 gel filtration chromatography and peptides were detected by UPLC-MS, employing a similar process by using the supernatant which was mixed with sodium phosphate buffer (pH 6) with a solid-to-solvent ratio of 1:1 (w/v) for 24 h with continuous stirring along with papain at (pH 6.0, 37˚C) and trypsin at (pH 8.0, 40 °C) at a total enzyme dose of 2.5%. GFC had also been used for the purification of marine derived peptides from rotifer (Byun et al. 2009) and sardinelle (Bougatet et al. 2010). The current study was carried out using lyophilized sample of A. membranaceus having a protein concentration of about 3.2 mg/mL. Vennila et al. (2011) studied the protein concentration of Octopus vulgaris was estimated to be about 144 µg/mL, and the molecular weight profiling of the protein bands from ink sample of Octopus sp., ranging from 82 to 248 kDa. Similarly, the crude sample of *O. aegina* and *O. dolfusii* revealed prominent bands from 32 to 72 kDa respectively (Monalisa et al. 2013). Protein recoveries from cuttlefish viscera using the soluble fraction after hydrolysis, i.e., Protamex resulted in 57.2% of protein recovery, while with Alcalase, up to 64.3% of proteins were recovered (Emna et al. 2009).

Two purified proteins G-6 and G-4-2 were obtained from *Arca subcrenata* Lischke using the homogenization, salting-out with ammonium sulfate, ion-exchange chromatography and gel filtration chromatography techniques. The molecular weight of G-6 and G-4-2 were detected as 8.2 kDa and 16 kDa after purification. G-6 and G-4-2 were found to inhibit the proliferation of human tumour cells in vitro (Liyan et al. 2008). On accounting, above said research, purification enhances activity and also provided different sizes of proteins proved through SDS-PAGE and IEF-PAGE. The present study is based on studying the anticancer property from the Posterior salivary gland (PSG) of *A. membranaceus*, further assessing anticancer activity in the HeLa cell lines (cervical cancer cell lines) of humans. In vitro study was performed using three doses of OM-H at different concentrations on cancer cell lines namely control, 150 µg/mL, 200 µg/mL and 250 µg/mL. It was found that 250µg/mL dose showed the highest toxicity of 45.01% and the IC50 was calculated to be 405µg/mL. Since the sample concentration and inhibition rates were moderate we can still increase the concentration of the sample with reference to the IC50 value.

Additionally, two proteins were detected after fractionating by gel permeation chromatography has a molecular weight of 20 kDa and 25 kDa. According to an in vivo study of Apoptosis in Ehrlich's Ascites carcinoma of mice using Posterior salivary gland of *Octopus aegina* by Karthigayan et al. 2007, four groups of animals were treated with three doses of venom 15, 75, and 150 µg/kg of body weight with standard drug 5 fluorouracil (20mg/kg/b.w), after treatment for 10 days the lifespan, tumor volume, hemoglobin, red blood cells, and lymphocytes were significantly dropped over normal cell body weight, neutrophils, and viable tumor cells count were increased. As per a study conducted by Aleman et al. (2011), squid gelatin didn't show the cytotoxic or anti-proliferative result on cancer cells. However, the hydrolysates from squid gelatin affected the viability of each cell lines otherwise relying on the enzymes used. Cell viability was definite on 24, 48 and 72 h with an amendment of medium and also the addition of 1 mg/mL concentration of the product at respective time intervals. Jumeri and Kim (2011) reported that anti cancer activity from crude hydrolysates and fractions of solitary tunicate. It was shown that activity of crude was weak comparing the fractions obtained from the same hydrolysate were crude hydrolysates has shown 16.5–28.4% for crude hydrolysate 15.2–30.5% for F1 and 21.7–51.5% for F2 at 100–1000 µg/mL against HeLa cancer cell lines.

Interestingly, in another study done by Ding et al. (2011) the sepia ink oligopeptides prepared using the protease trypsin from Sepia ink was found to inhibit the growth of DU-145 cells whereas sepia ink oligopeptides also caused a linear decrease in cell viability on a dose-dependent manner. The anti-tumor activity of a new type of peptidoglycan isolated from squid ink was shown to have a cure rate of 64% for Meth A tumor from BALB/c mice. One-fifth of the tumor-bearing mice was cured with 3 injections (1 mg/head) of the acetone delipidated squid ink or a prolongation of survival was observed in the treated animals (Sasaki et al. 1997). Recent studies stated high toxic diversity with coleoid cephalopod (Ruder et al. 2013). Sivaramakrishnan et al. (2019) investigated a study on the effect of nudibranch *K. ornata* on colon cancer (SW620) cell lines with three different solvents. The inhibition rates were 38.63% using 250 µg/mL of acetone extract and 18.44% and 14.35% using ethyl acetate and methanol extracts respectively. These rates were found to be slightly lower than the current study.

Conclusion

The salivary glands of octopus are associated with buccal mass and mandible. The posterior salivary gland contains certain bioactive substances such as tyramine, histamine, octopamine, etc. The purified sample of posterior salivary gland (PSG) of A. *membranaceus* displayed anticancer activity against HeLa cell lines of cervical cancer. Significant cytotoxicity levels were observed at varying concentrations. Therefore, it can be concluded that the PSG of *A. membranaceus* can function as an effective anticancer agent. Further studies on characterization and purification of the components could elucidate a good source of anticancer agents which could replace the costliest antibiotics and could alter into a targeted anticancer drug.

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Compliance with Ethical Standards

Conflict of interest All the authors declare that there is no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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